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权利要求书 2 页 说明书 16 页 附图 6 页

[54] 发明名称 长效生长激素及药物组合物

[57] 摘要

本发明公开了一种采用大分子量聚乙二醇修饰生长激素的方法，得到了能够显著延长生长激素半衰期的修饰产物，此修饰产物的结构单一，生产工艺简单，可进行严格质量控制，具有强的实用性。

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1. 高纯度的聚乙二醇生长激素偶联物，其中聚乙二醇和生长激素的摩尔比是 1:1；
2. 权利要求 1 中的偶联物，其中聚乙二醇的分子量在 20,000 到 120,000 之间；
3. 权利要求 2 中的偶联物，其中聚乙二醇的分子量在 40,000 到 80,000 之间；
4. 权利要求 1 中的偶联物，其中聚乙二醇含有两个到四个分叉支链；
5. 权利要求 4 中的偶联物，其中每个聚乙二醇支链的分子量在 5,000 到 20,000 之间；
6. 权利要求 1 中的偶联物，其中偶联物的纯度大于 95%；
7. 权利要求 1 中的偶联物，其中偶联物能起到长效作用；
8. 一种生产权利要求 1-7 偶联物的方法，其特点是单功能团活化的聚乙二醇与生长激素在 pH 值 5.5 到 7.5 之间的水溶液进行偶联反应；
9. 一种生产权利要求 8 偶联物的方法，其特点是单功能团活化的聚乙二醇与生长激素在 pH 值 6.5 到 7.0 之间的水溶液进行偶联反应；
10. 一种生产权利要求 1-7 中偶联物的方法，其特点是将偶联混合物用 Superdex 75 分子筛层析进行分离纯化；
11. 一种生产权利要求 1-7 中偶联物的方法，其特点是将偶联混合物用 阴离子交换层析柱进行分离纯化；
12. 一种根据权利要求 11 的方法，其中阴离子交换柱是 Q；
13. 一种根据权利要求 12 的方法，其中权利要求 1-6 的偶联物在含 50 毫摩尔氯化钠的缓冲液中洗脱；
14. 一种含有权利要求 1-7 中偶联物的有效剂量的药物组合物，以及药物接受的稀释剂、稳定剂或其它辅料；
15. 一种根据权利要求 14 的药物组合物，其特点是此药物组合物具有长效的功能；
16. 一种高分子的分叉型聚乙二醇分子，结构式表达为：

    Rx-B-R1

    其中 R 为连在连接物 B 上的聚乙二醇支链，x 为此支链的数目，在 2-4 之间，R1 是个双功能聚乙二醇分子，一头连接在连接物 B 上，另一头具有游离的羧基或羟基；
17. 一种根据权利要求 16 的高分子的分叉型聚乙二醇分子，其分子量在 20,000 到

120,000之间；

18. 一种根据权利要求17的高分子的分叉型聚乙二醇分子，其分子量在40,000到80,000之间；

19. 一种聚乙二醇偶联物，其特点是权利要求16到18的聚乙二醇分子与蛋白质或其他分子形成偶联物。

长效生长激素及药物组合物

技术领域

本发明属生物制剂技术领域。具体涉及长效生长激素，其制备方法，功能和用途。

背景技术

人生长激素是人脑垂体分泌的蛋白质激素，是人体正常生长发育所不可缺少的调节激素。生长激素最早的来源是从人脑垂体提取，但由于来源有限，且质量保证困难，此产品难以广泛使用。基因工程技术的出现，使得人生长激素的大规模生产成为可能，而重组人生长激素成为生物工程制药行业的第二项产品而得到广泛应用，目前主要用于侏儒症、大面积创伤恢复、成人生长激素缺乏症、艾滋病人瘦弱恢复等。

由于人生长激素是蛋白质，其体内半衰期低于 2 小时，因此用药手段主要局限于频繁的注射方法，常规方法为每天注射，用药周期在六个月到一年，这种频繁的注射，给病人带来严重的不便，并且提高了用药成本。因此，改善生长激素的用药方法，减少用药频率，降低用药成本，提高病人医从性，增加疗效的重要课题。

美国 Genentech 公司与 Alkermers 公司合作开发了用 PLGA 包埋技术形成的释放微粒，可以将用药频率由每天注射降低一次到每个月注射一次，并得到 FDA 批准上市。但此产品生产工艺复杂，疗效比原产品稍差。

利用聚乙二醇修饰蛋白质，可以延长其半衰期，减少免疫原性，增加稳定性，是一项已成功得到应用的技术。目前利用此技术开发的产品，已经上市的包括 PEG-门冬酰胺酶，PEG-ADA（腺苷脱氨酶），PEG-干扰素和 PEG-GCSF（粒细胞集落刺激因子）。其中 PEG-干扰素 由原来的每 2 天或每天注射一次降低到每周注射一次，而且疗效显著改善。PEG-GCSF 也由每天注射一次改善为每个化疗疗程注射一

次。

Clark 等(J.Biol.Chem.，271:21969-21977，1996)对聚乙二醇修饰生长激素进行了研究，并得到了能够将生长激素半衰期延长到超过15 小时以上的产品，在去除脑垂体的大鼠模型中，注射频率可以降低到每 5 天注射一次。此研究采用分子量为 5000 的聚乙二醇，因此需要多个聚乙二醇分子修饰才能达到理想的半衰期延长效果，但过度修饰对生长激素的活性影响较大，此研究表明，5 个聚乙二醇分子修饰的效果最理想。尽管这种方法证明了用聚乙二醇修饰生长激素的可行性，但是由于活性的降低，可能需要增加剂量来补偿，而产品的不均一性使得大规模生产时工艺和质量控制难以保证，因此，此项研究的实用性不强。

发明内容

本发明要解决的问题是提供一种采用大分子量聚乙二醇修饰生长激素的方法，从而得到能够延长生长激素半衰期的修饰产物。

本发明解决技术问题的方案是：

1、聚乙二醇和人生长激素的偶联物及生产方法

生长激素在体内的半衰期取决于多方面的因素，如蛋白质的稳定性、结合蛋白的结合和分离、非特异性吸附和降解、抗体的形成、肾脏排除、与受体结合并内吞等等，这些因素决定了生长激素在体内的分布、停留时间和代谢，同时也决定了外源性生长激素的药物动力学特点。将蛋白质与亲水性的高分子如聚乙二醇偶联，可增加蛋白稳定性、减少非特异性吸附和抗原性，达到一定分子量时，可大大降低肾脏的排除效率，是延长蛋白质药物体内半衰期的有效方法。

人生长激素的分子量为 20kDa，含有 10 个一级氨功能团，其中 1 个阿拉法氨基，9 个为赖氨酸侧链氨基。阿拉法氨基和 140 位的赖氨酸侧链均非常活跃，可以与活化的羧基反应形成肽键。N-羟基琥珀酰胺 (NHS)活化聚乙二醇分子是目前大分子偶联常用的方法。

Clark 等人(J. Biol. Chem.，271:21969-77，1996) 采用了分子量为 5000 的聚乙二醇与人生长激素偶联，结果表明多个聚乙二醇分子可以联接到人生长激素分子上，随着联接聚乙二醇分子数目增大，分子量增大，偶联物半衰期也延长，但同时多个聚乙二醇分子联接后对生

长激素活性造成严重负面影响，聚乙二醇分子联接越多，活性损失就越大。这种半衰期延长或活性损失的中和结果使作者得出结论为每个生长激素分子联接五个聚乙二醇分子得到最理想的长效结果。

人生长激素的半衰期取决于多项因素，其中肾脏排除是重要的一项，为了减少肾脏排除，分子量要超过一定的临界值(大约 70kDa)，因此要偶联大分子聚乙二醇。而为了减少活性损失，最好只偶联一个聚乙二醇分子。这种方法在长效干扰素中已得到成功应用(Bailon et al.，Bioconjugate Chem.，12:195-202，2001)。要做到只在生长激素分子偶联一个聚乙二醇分子，较可能的位点是阿拉法氨基，其 pKa 在 7.6-8.0（比赖氨酸侧链氨基的 pKa(10.0-10.2)要低）。 采用醛基与阿拉法氨基在低pH偶联的方法可以特异地将聚乙二醇偶联在G-CSF蛋白的N端(Kinstler et al.，US Patent 5985265，1999； Kinstler et al.，Pharm. Res.，13:996-1002，1995)。但在同样条件下，生长激素的偶联效率很低。

本发明一方面是提供一种生长激素与高分子聚乙二醇的偶联物，二者的摩尔比为 1:1，另一方面是提供其相应的偶联方法，这种方法是将生长激素与 NHS 活化后的聚乙二醇在缓冲液中混合反应并形成偶联物。

用 NHS 活化的聚乙二醇分子量可以是 20,000 到 120,000，更理想的是 40,000 到 80,000 之间。所有聚乙二醇可以是线性的，也可以是分叉的。活化的 40kDa 分叉型聚乙二醇(mPEG2-NHS)购自美国 Shearwater 公司，80kDa 分叉型聚乙二醇(mPEG4-NHS)及活化方法在本发明中公布。缓冲体系、pH、摩尔比例、反应温度和时间等是反应过程重要的控制条件。磷酸缓冲液为本发明首选缓冲体系，其它缓冲液，如碳酸、柠檬酸、琥铂酸等、只要满足 pH 在 6 至 7.5 之间有足够的缓冲能力，并对偶联反应没有干扰也可以使用。缓冲液浓度在 50-200mM 之间。

传统的 NHS 偶联方法为了增加偶联效率，一般选择偶联缓冲液的 pH 控制在 7.5 到 9.0 之间。为了增强选择性，我们在偶联生长激素时将 pH 降低到 6.0 到 7.5 之间，更理想的是 6.5 到 7.0 之间。在这种条件下，一般认为 NHS 偶联方法效率很低。聚乙二醇与生长激素

的摩尔比可以在 1:1 到 10:1 的范围内，更为适用的范围是 3:1 到 7:1。反应 pH 对摩尔比有一定影响，pH 越高，则需要的聚乙二醇与生长激素的摩尔比可以降低。反应的温度和时间有一定关系，一般在室温条件下一个小时反应可以完成，而在 4℃下，一般采用过夜反应。在本发明所采用的低 pH 条件下，NHS 活性基团的水溶液稳定性大大增加，因此时间延长有助于反应完全。此外，蛋白质在低温下一般具有更好的稳定性。因此，首选的条件是 2-8℃过夜反应。满足上述条件后，聚乙二醇与生长激素偶联反应的主要产物是一个聚乙二醇与一个生长激素分子偶联。尽管确切的偶联位点并没有通过试验证实，在这些条件下，最可能的偶联位点是 N-端的阿拉法氨基。

本发明的另外一个方面是提供了纯化聚乙二醇生长激素偶联物的方法。在本发明条件下需要与偶联物分离的主要组份是未偶联的生长激素，两者之间的差别为一个聚乙二醇分子，由于聚乙二醇的分子量在 20,000 以上，采用 Superdex 75 (Pharmacia Biotech)的分子筛柱将偶联物与未偶联生长激素分离，偶联物在排空体积出现，而未偶联生长激素具有更长的滞留时间。进行层析时对缓冲液和 pH 限制不多，可根据下一步工艺需要进行调整。

本方法可以有效地将偶联物与未偶联生长激素分离，但不能将游离的聚乙二醇与偶联物分开。对于其他与生长激素分子量相近的蛋白质，如红细胞生长素、G-CSF、干扰素、GM-CSF、白介素等，可以采用同样的方法。需要满足的条件是聚乙二醇分子量在 20,000 以上，蛋白质分子量低于 40,000。

本发明的另一方面是提供了同时将聚乙二醇生长激素偶联物与未偶联生长激素和聚乙二醇分离的方法。这种方法是将偶联反应后的产物首先置换到最低离子强度的阳离子性缓冲液中，如 Tris，然后将反应产物在阴离子交换柱中分离并用不同离子强度的缓冲液洗脱。在此条件下，游离聚乙二醇结合能力很低，首先被分离，聚乙二醇生长激素偶联物与阴离子柱的结合能力与游离生长激素相比降低，因此在较低的离子强度条件下洗脱，而游离的生长激素在较高的离子强度下洗脱。更为具体地说，偶联反应混合物在反应完成后首先用脱盐层析，如 Sephadex G25，进行缓冲液交换，适用的缓冲液体系为阳离子缓

冲液， 如 Tris。缓冲液 pH 值应该高于 6.5，一般采用 pH7.4，浓度通常为 20mM。将缓冲液置换后的反应混合物在 Q 阴离子交换柱(如 Mono-Q 或 Q-Sepharose，Pharmacia Biotech)上进行纯化，游离聚乙二醇在上样和用平衡液洗涤时便被分离，而聚乙二醇生长激素偶联物和游离生长激素则结合在柱上。上样后，用与上样相同的平衡液(20mM Tris，PH7.4)洗涤，然后用同样的缓冲体系，逐步增加离子强度来洗脱。聚乙二醇生长激素偶联物与阴离子柱结合的能力被减弱，因此在含有 50mM 氯化钠的洗脱液(50mM 氯化钠，20mM Tris，PH7.4)中洗脱聚乙二醇生长激素偶联物。未偶联生长激素在超过 65mM 氯化钠的洗脱液(并含有 20mM Tris，PH7.4)中洗脱。本方法可以在同一步有效地将游离聚乙二醇和生长激素从需要的偶联产物中分离出来，得到高纯度的适于药用的聚乙二醇生长激素偶联物。

1. 药物组合物

去除大鼠脑垂体造成人为的生长激素缺乏症。如果不给外源性生长激素，去除脑垂体的大鼠体重将保持不变，此模型是检测生长激素生物活性的标准方法。人生长激素在大鼠中的半衰期小于 30 分钟(Jorgensen et al. Pharmacol.Toxicol.，63:129-134，1988)，要观察人生长激素的活性需要给每天大鼠注射人生长激素。长效效果是指延长了生长激素的生物学活性，这种长效结果不能通过简单的剂量增加来获得。例如，在脑垂体去除大鼠中，增加生长激素的剂量可以相应增加体重增加的程度，但无论剂量如何，其增重效应不超过 24 小时。长效的生长激素则应该在同样或更少的剂量下，在单位时间内用药次数减少的条件下，达到同样的体重增加的目的。

本发明提供了治疗哺乳动物各种疾病的方法。方法包括给需要治疗的哺乳动物提供本发明中有效剂量的聚乙二醇生长激素偶联物。这种偶联物可以用于治疗生长激素缺乏症或者使用生长激素后可以产生有利效果的病症。用于治疗上述病症所需聚乙二醇生长激素偶联物的剂量一般取决于偶联物所含生长激素的活性。这种剂量的要求是能够产生有效的和有利的临床反应，其最大剂量取决于药物不产生临床上重要的副作用。一般来讲，所用偶联物的剂量可以单位时间内累计用常规生长激素的剂量为参考。这种参考剂量只是用于说明目的，最

佳剂量的选定要根据临床经验和治疗适应症具体确定。本发明的高纯度的聚乙二醇生长激素偶联物可以用于生产可用于治疗哺乳动物的药物组合物。这种药物组合物可以是溶液、悬浮液、冻干粉剂、片剂、胶囊等等常规的药物剂型，用药方法主要采用非肠胃给药方法，但口服或呼吸剂型也可使用。

2. 高分子量分叉型聚乙二醇的合成

本发明的另外一个方面是提供一种合成高分子量分叉型的聚乙二醇分子的方法。这种聚乙二醇的分子定义为下列结构代表：

Rx-B-R1

其中 R 是接在分叉连接物 B 上的聚乙二醇支链，可以是直线型分子，也可以是分叉型分子。X 为在分叉连接物上的聚乙二醇支链数目，可以是在 2-4 之间。聚乙二醇支链可以是同样大小或形状，也可以是不同大小或形状，分子量可以在 2000 到 20,000 之间。

B 是含有至少两个亲核功能团的分叉连接物，并通过亲核基团的反应与聚乙二醇支链相邻。B 并与另外一个聚乙二醇支链 R1 相连。亲核基团可以是氨基，另一基团可以是羧基或羟基。赖氨酸是连接物 B 的典型代表。

R1 是一个线性的具有不同功能团的聚乙二醇，其中一头连接在 B 连接物上，另一头为可后继活化的集团，如羧酸或羟基。R1 的分子量在 200 到 20,000 之间。

合成时，首先将 R1 支链与活化的 B 反应，形成分叉，反应物 B 的亲核基团采用同样或不同的保护基团保护。以赖氨酸为例，如采用同样的保护基团，如 Fmoc-Lys(Fmoc)或者 Boc-Lys(Boc)，在保护基团去除后，可以连接相同大小或形状的聚乙二醇分子。如采用不同的保护基团，如 Fmoc-Lys(Boc)或相反的保护，可连接不同大小或形状的聚乙二醇分子。B 的活化采用形成对硝基苯基（p-nitrophenyl）或琥珀酸亚胺基（ succinimidyl） 功能团，并与 R1 的亲核基团反应。

在反应完成并将保护基团去除后，所得产物 B-R1 的结构为一头具有两个亲核基团(氨基)，另一头带有羧酸或羟基以便最终活化。

聚乙二醇支链 R 一般为甲氧基-聚乙二醇，可以用常规方法活化，活化的聚乙二醇也可以从 Shearwater 公司购买商业产品。对硝基苯基

或NHS活化后的聚乙二醇可以与B-R1上的亲核基团(氨基)反应，形成共价键相连接的稳定的终产物Rx-B-R1。

本发明与文献中其它形成分叉聚乙二醇的方法相比(Monfardini et al., Bioconjugate Chem., 6:62-69, 1995; Nathan et al., Macromolecules, 25:4476-84, 1992)，优点在于所有的反应都在有机溶剂中进行，以利反应效率的提高。反应产物在与蛋白质连接时，在蛋白质反应位点和大分子之间拥有空间，利于反应完成，同时侧链的数目、大小和形状可以自由选择。

本发明公开了一种采用大分子量聚乙二醇修饰生长激素的方法，得到了能够显著延长在大鼠中疗效的修饰产物，此修饰产物的结构单一，生产工艺简单，可进行严格质量控制，具有强的实用性。

附图说明

图1：人生长激素及聚乙二醇偶联物在脑垂体去除大鼠中的增重效果

脑垂体去除大鼠用溶媒(AQ缓冲液，实心正方)、每天10微克人生长激素(空心正方)、每7天70微克PEG40-hGH偶联物(实心圆圈)和每7天70微克 PEG80-hGH偶联物(空心圆圈)治疗。图中显示为治疗后每天体重增加的平均值，数据表示为平均值±标准方差。

图2：人生长激素聚乙二醇偶联物在脑垂体去除大鼠中的增重效果的量效关系

脑垂体去除大鼠用溶媒(AQ缓冲液，实心正方)，每天10微克人生长激素(空心正方)，每7天30微克PEG80-hGH偶联物(实心圆圈)和每7天70微克 PEG80-hGH偶联物(空心圆圈)治疗。图中显示为治疗后每天体重增加的平均值，数据表示为平均值±标准方差。

图3：人生长激素聚乙二醇偶联物的SDS-聚丙烯酰胺凝胶电泳

4-20% SDS-聚丙烯酰胺凝胶预制胶在电泳完成后用考马斯亮蓝染色后照相，本图为染色后胶的照片。

第1道，人生长激素；

第2道，比例为1:3的人生长激素:聚乙二醇反应产物；

第3道，比例为1:5的人生长激素:聚乙二醇反应产物；

第4道，比例为1:7.5的人生长激素:聚乙二醇反应产物；

第 5 道，比例为 1:10 的人生长激素:聚乙二醇反应产物；

第 6 道，低蛋白质分子量标准；

第 7 道，Mono-Q 柱 50mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:3)；

第 8 道，Mono-Q 柱 65mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:3)；

第 9 道，Mono-Q 柱 500mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:3)。

图 4：人生长激素聚乙二醇偶联物的 SDS-聚丙烯酰胺凝胶电泳

4-20%SDS-聚丙烯酰胺凝胶预制胶在电泳完成后用考马斯亮蓝染色后照相，本图为染色后胶的照片。

第 1 道，Mono-Q 柱 50mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:5)；

第 2 道，Mono-Q 柱 65mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:5)；

第 3 道，Mono-Q 柱 500mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:5)；

第 4 道，Mono-Q 柱 50mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:7.5)；

第 5 道，Mono-Q 柱 65mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:7.5)；

第 6 道，Mono-Q 柱 500mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:7.5)；

第 7 道，Mono-Q 柱 50mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:10)；

第 8 道，Mono-Q 柱 65mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:10)；

第 9 道，Mono-Q 柱 500mM 氯化钠洗脱峰(人生长激素:聚乙二醇比例 1:10)；

第 10 道，低蛋白质分子量标准。

图 5：人生长激素及 40KDA 聚乙二醇人生长激素偶联物在脑垂

体去除大鼠中的增重效果

脑垂体去除大鼠用溶媒(AQ 缓冲液，实心正方)，每天 20 微克人生长激素(空心圆圈)，每 7 天 70 微克 PEG40-hGH 偶联物(实心圆圈)和每 7 天 140 微克 PEG80-hGH 偶联物(空心正方)治疗。图中显示为治疗后每天体重增加的平均值，数据表示为平均值±标准方差。

图 6：人生长激素及聚乙二醇偶联物同剂量一次注射在脑垂体去除大鼠中的增重效果

脑垂体去除大鼠每天用 140 微克人生长激素(实心圆圈)和每 7 天用 140 微克 PEG40-hGH 偶联物(空心圆圈)注射一次治疗。图中显示为治疗后每天体重增加的平均值，数据表示为平均值±标准方差。

具体实施方式

实施例 1 高分子量分叉型聚乙二醇的制备

为延长生长激素的半衰期，高分子量分叉型聚乙二醇较为适用。以下叙述了一种合成多分叉分子量为 80,000 的聚乙二醇的方法，而这个方法同样可用于合成其他不同分子量或形状的聚乙二醇。

将 11.8mg Fmoc-Lys(Fmoc)-OH (MW590，20μmol， Advanced ChemTech)溶解在 120μl 含有 24μmol NHS(Sigma)的二甲基甲酰胺(DMF)中，加入 3.8μl 二异丙基碳二亚胺 (DIC，MW126， d=0.8，24μmol， Advanced ChemTech)，反应在室温下振荡两小时。将 68mg NH2-PEG-COOH (MW3400， 20μmol， Shearwater)溶解在 100μl 二氯甲烷(DCM)中，加入 6.9μl 二异丙基乙胺 (DIPEA，MW129，d=0.74，40μmol，Sigma)，并与前部反应混合，室温下振荡一小时后，加入 10%六氢吡啶，室温下振荡 5 分钟，将反应产物以超过体积比 1:10(混合物：乙醚)的比例沉淀在乙醚中并真空干燥。得到 78mg $(NH_2)_2$-PEG-COOH 终产品。

将 8.8mg $(NH_2)_2$-PEG-COOH (5μmol 氨基)溶于 100μl DCM，然后加入 200mg $mPEG_2$-40K-NHS (MW42500，5μmol，Shearwater)和 1.7μl DIPEA (10μmol)，反应在室温下振荡一夜。产物在乙醚中沉淀并真空干燥。

合成的 80kDa 聚乙二醇在 Superdex 200 层析柱上纯化(2.6×40cm)，将柱用水平衡，并将要纯化的产物溶于 5ml 水中。上样后用

水洗脱，并在214nm观察，并将第一个峰混合，真空干燥。

纯化后的80kDa聚乙二醇采用DIC进行活化。将100mg 80kDa $mPEG_4$-COOH (1.25μmol)溶于0.5ml干燥DCM中，加入1.3μmol NHS和0.25μl DIC (1.5μmol)。反应在室温下振荡过夜。将产物用乙醚沉淀并真空干燥。回收产物103mg，干燥保存在-20℃。

实施例2 生长激素与80kDa和40kDa聚乙二醇偶联

将234mg生长激素冻干粉剂溶于4ml 0.1M磷酸钠，pH 7.0。将HiTrap Sephadex G25脱盐柱(2.5×15cm，Pharmacia Biotech)用人0.1M磷酸钠（pH 7.0）平衡，并将生长激素样品上样，在280nm观察，收集第一个峰(18ml)。测定280nm光密度并用消光系数0.68计算生长激素浓度，结果为280nm光密度1.2，蛋白质浓度1.76mg/ml，总蛋白32mg。

与80kDa聚乙二醇偶联时，称取60mg活化后的80kDa $mPEG_4$-NHS (0.7μmol)，加入到1.1ml含6mg生长激素(0.3μmol)的磷酸钠(pH7.0)溶液中，反应在4℃过夜振荡。

与40kDa聚乙二醇偶联时，称取60mg活化后的40kDa $mPEG_2$-NHS (1.5μmol)，加入到2ml含10mg生长激素(0.3μmol)的磷酸钠(pH7.0)溶液中，反应在4℃过夜振荡。

实施例3 80kDa和40kDa聚乙二醇生长激素偶联物用Superdex 75纯化

80kDa和40kDa聚乙二醇生长激素偶联物用Superdex 75层析柱(1.5×40cm， Pharmacia Biotech)纯化。将层析柱用150mM氯化钠，10mM柠檬酸钠，0.2%吐温20，pH 6.0的溶液平衡后上样，上样体积低于柱体积的5%，用280nm观察蛋白峰，并收集第一个洗脱峰。共收集了35ml浓度为0.16mg/ml的40kDa聚乙二醇人生长激素偶联物，总蛋白量5.6mg。共收集了28ml浓度为0.08mg/ml 80kDa聚乙二醇人生长激素偶联物，总蛋白量2.6mg。

实施例4 40kDa和80kDa聚乙二醇人生长激素偶联物在脑垂体去除大鼠中的长效效应

六十只八周龄脑垂体去除的雌性Sprague Dawley大鼠购买于Taconic Farms公司，四只大鼠因健康不佳剔除。分组前10天，观察

每个大鼠体重变化，并将体重增加超过所有小鼠平均值一个标准方差的大鼠剔除。其余大鼠(53 只)随机分为以下 5 组：

溶媒组，10 只大鼠，连续 14 天每天皮注 1 毫升 AQ 缓冲液(0.2% 吐温-20， 150mM 氯化钠，10mM 柠檬酸钠， pH 6.0)；

生长激素组，10 只大鼠，连续 14 天每天皮注 1 毫升含 10 微克人生长激素的 AQ 缓冲液；

80N-70 组，11 只大鼠，第 0 天和第 8 天皮注 1 毫升含 70 微克 80kDa 聚乙二醇人生长激素偶联物的 AQ 缓冲液；

80N-30 组，11 只大鼠，第 0 天和第 8 天皮注 1 毫升含 30 微克 80kDa 聚乙二醇人生长激素偶联物的 AQ 缓冲液；

40N-70 组，11 只大鼠，第 0 天和第 8 天皮注 1 毫升含 70 微克 40kDa 聚乙二醇人生长激素偶联物的 AQ 缓冲液。

每天测定大鼠体重，图 1 和图 2 显示了各组大鼠在治疗过程中体重变化的曲线，第 7 天和第 14 天各组大鼠体重变化的平均值及统计学分析列在表 1 和表 2 中。统计学分析采用 EXCEL 软件的 t 检验功能，指标为双尾和不等方差。

表 1 治疗后第 7 天脑垂体去除大鼠的平均体重增加

组	n	体重增加(g) (mean±sd)	P(比溶媒)	P(比生长激素)
溶媒	10	0.6±1.8		
生长激素	10	14.2±2.1	<0.0001	
40N-70	11	13.0±2.2	<0.0001	>0.05
80N-30	11	9.9±2.2	<0.001	<0.01
80N-70	11	13.2±2.0	<0.0001	>0.05

表2 治疗后第14天脑垂体去除大鼠的平均体重增加

组	n	体重增加(g) (mean±sd)	P(比溶媒)	p(比生长激素)
溶媒	10	1.0±2.7		
生长激素	10	19.8±2.8	<0.0001	
40N-70	11	20.0±3.2	<0.0001	>0.05
80N-30	11	14.5±4.1	<0.0001	<0.01
80N-70	11	19.5±4.4	<0.0001	0.05

结果表明，40N-70和80N-70组大鼠的平均体重增加与阳性对照生长激素组没有区别，即高分子量分叉型聚乙二醇生长激素的偶联物每7天一针，起到了常规生长激素每日注射同样的增重效果。这种增重效果与所用偶联物的剂量有量效关系。

实施例5 聚乙二醇与生长激素偶联及偶联物的纯化

将43.5mg人生长激素冻干粉剂溶于1.0ml水中，上样到HiTrap G25脱盐柱将缓冲液置换到50mM磷酸钠，pH6.5。测定280nm的光密度，并用0.68的消光系数计算蛋白浓度。回收的总生长激素量为5.7毫克，将其分为四份，每份含1.4mg蛋白，体积0.75ml。

按下表称取四份适量的mPEG2-NHS(MW40,000，Shearwater)，并分别加入到生长激素样品中，反应在4℃过夜进行。

PEG:hGH(摩尔比)	3:1	5:1	7.5:1	10:1
hGH (nmol)	71.62	71.62	71.62	71.62
PEG (mg/nmol)	8.6/214.9	14.3/358.1	21.4/537.2	28.6/716.2

以上反应完成后，分别采用HiTrap G25脱盐柱将缓冲液置换为20mM Tris，pH7.4。每个样品分别上样到已用20mM Tris， pH7.4缓冲液平衡的Mono-Q HR5/5层析柱(Pharmacia Biotech)，并用阶梯增加离子强度的方式进行洗脱，洗脱峰在280nm观察，洗脱液为：

洗脱液1:50mM氯化钠，20mM Tris，pH7.4；

洗脱液2:65mM氯化钠，20mM Tris，pH7.4；

洗脱液 1:500mM 氯化钠，20mM Tris，pH7.4。

将洗脱后的各洗脱峰收集，每个样品取 80 微升，加入 5 微升 5 倍的 SDS-聚丙烯酰胺凝胶样品处理缓冲液，在 100℃加热到总体积低于 30 微升，将所有样品上到 4-20%的预制的 SDS-聚丙烯酰胺凝胶电泳，电泳完成后用考马斯亮蓝染色并照相。图 3 和图 4 是电泳后的照片。

结果表明，生长激素和 40kDa 分叉型聚乙二醇(mPEG2-NHS)可有效形成一 个聚乙二醇与一个生长激素分子结合的偶联物。生长激素和聚乙二醇二者的摩尔数比例在 1:5-1:7.5 时偶联效率较高，而且有较少的多个聚乙二醇分子与生长激素偶联的现象，效果最佳。

阴离子交换柱 Mono-Q 可以有效地将聚乙二醇生长激素偶联物与游离人生长激素和游离聚乙二醇分离。游离聚乙二醇在上样和平衡液洗脱时就被分开，而聚乙二醇生长激素偶联物在 50mM 氯化钠的洗脱液中洗脱，游离的生长激素在 65mM 以上的氯化钠洗脱液中洗脱。

将从 Mono-Q 柱洗脱的所有 50mM 氯化钠洗脱峰混合，并用 HiTrap G25 脱盐柱将缓冲液交换到 AQ 缓冲液中（150mM 氯化钠，0.2%吐温-20，10mM 柠檬酸钠，pH6.0）。置换缓冲液后的样品用 0.22μm 滤器除菌过滤(CAMEO 25AS)并保存在 2-8°C 用于动物活性试验。

实施例 6 用阴离子交换柱制备聚乙二醇生长激素偶联物

将 88.4mg 人生长激素冻干粉剂溶于 1.0ml 水中，上样到两个连接的 HiTrap G25 脱盐柱将缓冲液置换到 50mM 磷酸钠，pH6.5。测定 280nm 的光密度，并用 0.68 的消光系数计算蛋白浓度。回收的总生长激素量为 14 毫克，体积 5.5 毫升，将体积用 50mM 磷酸钠，pH6.5 溶液调整到 8 毫升，分为八份，每份含 1.75mg 蛋白，体积 1ml。

按下表称取八份 26.25 毫克的 mPEG2-NHS(MW40,000，Shearwater)，并分别加入到生长激素样品中，反应在 4°C 过夜进行。

PEG:hGH(摩尔比)	7.5:1
HGH(nmol)	87.5/份
PEG(mg/nmol)	26.25/656.25/份

以上反应完成后，采用 HiTrap G25 脱盐柱将缓冲液置换为 20mM Tris， pH7.4，并将样品上样到已用 20mM Tris， pH7.4 缓冲液平衡的 Mono-Q HR5/5 层析柱(Pharmacia Biotech)，并用阶梯增加离子强度的方式进行洗脱，洗脱峰在 280nm 观察，洗脱液为：

洗脱液 1:50mM 氯化钠，20mM Tris，pH7.4；

洗脱液 2: 65mM 氯化钠，20mM Tris，pH7.4；

洗脱液 1:500mM 氯化钠，20mM Tris，pH7.4。

将洗脱后的各洗脱峰收集，每个样品取 80 微升，加入 5 微升 5 倍的 SDS-聚丙烯酰胺凝胶样品处理缓冲液，在 100°C 加热到总体积低于 30 微升，将所有样品上到 4-20%的预制的 SDS-聚丙烯酰胺凝胶电泳，电泳完成后用考马斯亮蓝染色并照相。

结果表明，阴离子交换柱 Q-Sepharose Fast Flow 与 Mono-Q 柱的分离效果相同，聚乙二醇生长激素的偶联物在含 50mM 氯化钠的洗脱液中洗脱。

将从 Q-Sepharose 柱洗脱的所有 50mM 氯化钠洗脱峰混合，并用 HiTrap G25 脱盐柱将缓冲液交换到 AQ 缓冲液中（150mM 氯化钠，0.2%吐温-20， 10mM 柠檬酸钠，pH6.0）。置换缓冲液后的样品用 0.22μm 滤器除菌过滤(CAMEO 25AS)并保存在 2-8°C 用于动物活性试验。

实施例 7 聚乙二醇生长激素偶联物在脑垂体去除大鼠中的活性

四十只 8 周龄脑垂体去除雌性 Sprague Dawley 大鼠购买于 Taconic Farms 公司，两只大鼠因健康不佳剔除。分组前 12 天，观察每个大鼠体重变化，并将体重增加超过所有小鼠平均值一个标准方差的三只大鼠剔除。其余大鼠(35 只)随机分为以下 5 组：

溶媒组，9 只大鼠，连续 14 天每天皮注 1 毫升 AQ 缓冲液（0.2%吐温-20， 150mM 氯化钠，10mM 柠檬酸钠，pH 6.0）；

生长激素组，6 只大鼠，连续 14 天每天皮注 1 毫升含 20 微克人

生长激素的 AQ 缓冲液；

PEGhGH-70 组，10 只大鼠，第 0 天和第 7 天皮注 1 毫升含 70 微克 40kDa 聚乙二醇人生长激素偶联物的 AQ 缓冲液；

PEGhGH-140 组，10 只大鼠，第 0 天和第 7 天皮注 1 毫升含 30 微克 80kDa 聚乙二醇人生长激素偶联物的 AQ 缓冲液。

每天测定大鼠体重，图 5 显示了各组大鼠在治疗过程中体重变化的曲线，第 7 天和第 14 天各组大鼠体重变化的平均值及统计学分析列在表 3 和表 4 中.统计学分析采用 EXCEL 软件的 t 检验功能，指标为双尾和不等方差。

表 3 治疗后第 7 天脑垂体去除大鼠体重增加的平均值

组	n	体重增加(g) (mean±sd)	P（比溶媒）	P（比生长激素）
溶媒	9	1.93±2.10		
生长激素	6	17.07±1.94	<0.0001	
PEGhGH-70	10	12.99±2.66	<0.001	<0.05
PEGhGH-140	10	16.21±1.56	<0.0001	>0.05

表 4 治疗后第 14 天脑垂体去除大鼠体重增加的平均值

组	n	体重增加(g) (mean±sd)	P(比溶媒)	P(比生长激素)
溶媒	9	2.77±1.66		
生长激素	6	27.12±2.95	<0.0001	
PEGhGH-70	10	20.79±3.00	<0.0001	<0.001
PEGhGH-140	10	28.59±2.81	<0.0001	>0.05

结果表明，经 140 微克聚乙二醇生长激素偶联物(PEGhGH-140 组)每 7 天用 药一次的增重效果与每天注射 20 微克常规生长激素相当，并且大鼠的增重与所用偶联物剂量呈量效关系。

实施例 8 在脑垂体去除大鼠中同剂量一次用药的生长激素和聚乙二醇生长激素偶联物药效比较

九只 11 周龄雌性脑垂体去除的 Sprague Dawley 大鼠购自 Taconic Farms 公司，并随机分为以下两组：

生长激素组，4 只大鼠，一次皮注 1ml 含 140μg/ml 人生长激素的 AQ 缓冲液；

PEG-hGH 组，5 只大鼠，一次皮注 1ml 含 140μg/ml 聚乙二醇人生长激素偶联物的 AQ 缓冲液；

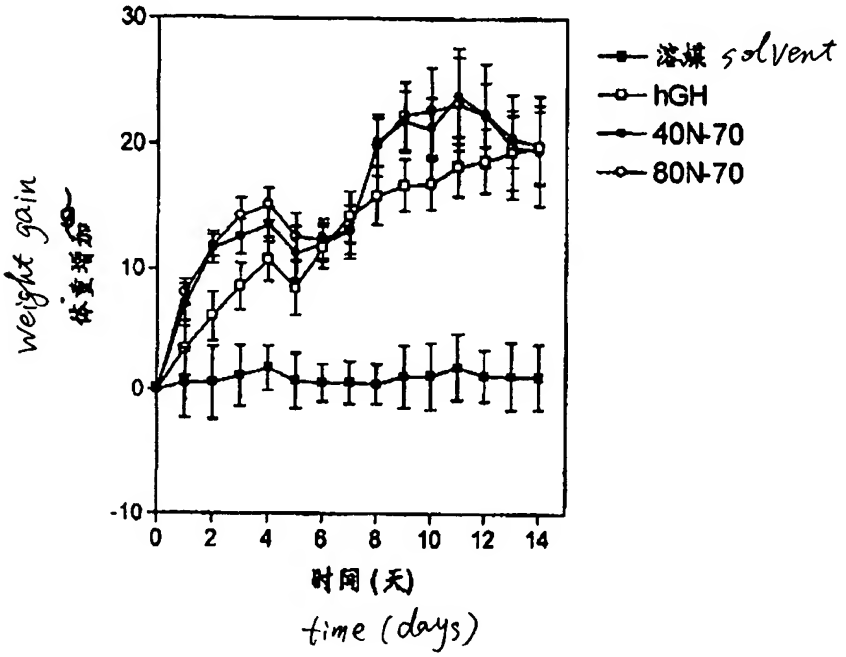
各组大鼠每天体重增加的变化曲线显示在图 6 中，第 7 天各组大鼠体重增加的平均值列在表 5 中。

表 5.治疗后第 7 天脑垂体去除大鼠体重增加的平均值

组	n	体重增加(g) (mean±sd)	p
生长激素	4	3.43±1.79	
PEGHGH	5	16.28±1.22	<0.0001

结果表明，采用 140 微克聚乙二醇人生长激素偶联物治疗的大鼠体重增加显著高于用 140 微克常规人生长激素组，即聚乙二醇人生长激素达到的长效目标，单纯增加生长激素剂量无法达到同样效果。

图 1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20
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0
-10
0
2
4
6
8
10
12
14
weight gain
体重增加
时间(天)
time (days)
溶媒 solvent
hGH
40N-70
80N-70

图 2

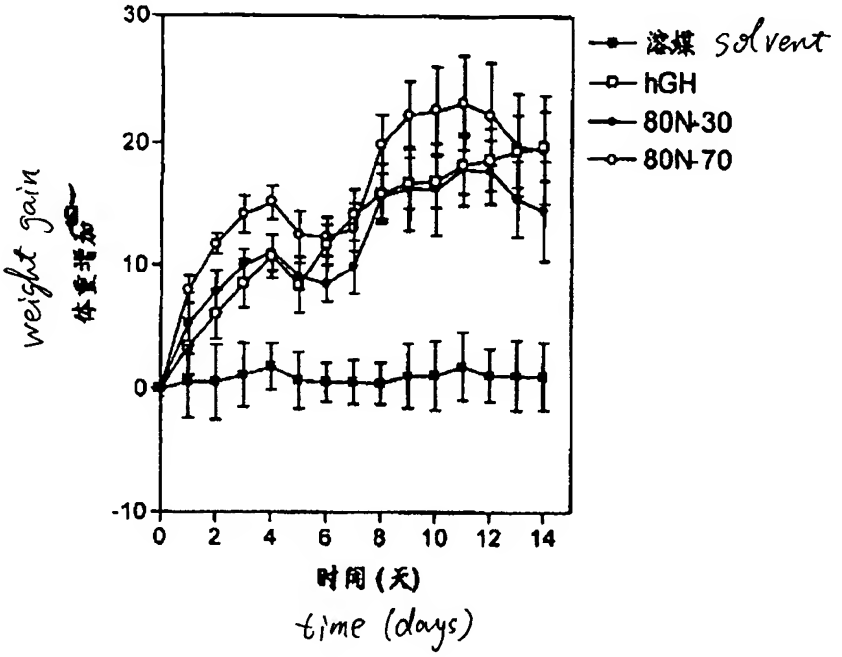

30
20
10
0
-10
0
2
4
6
8
10
12
14
weight gain
体重增加
时间(天)
time (days)
溶媒 solvent
hGH
80N-30
80N-70

图 3

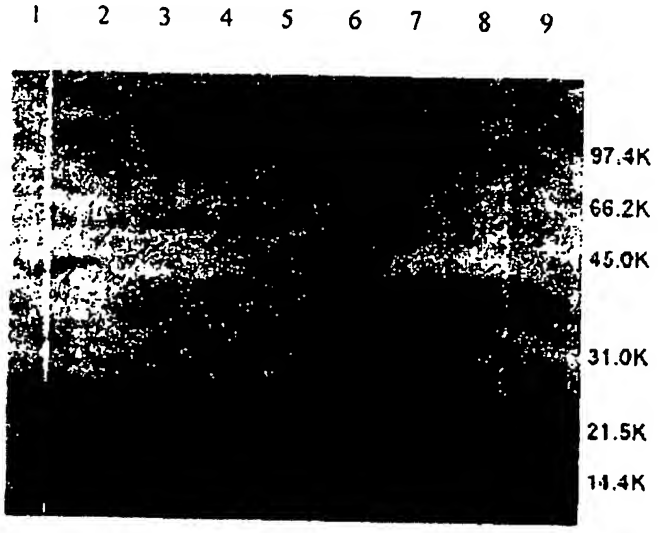


图 4

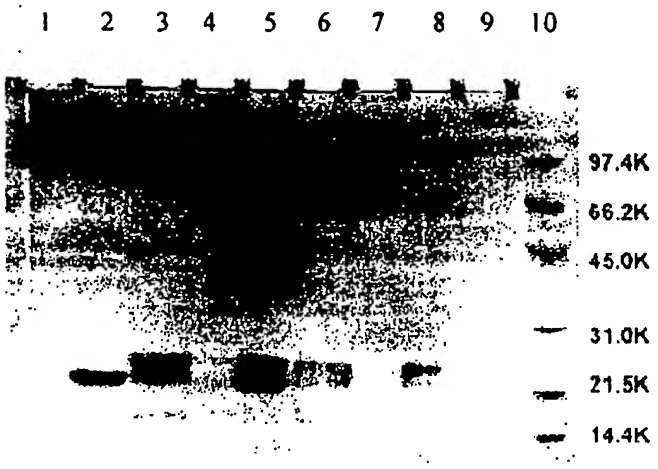


图 5

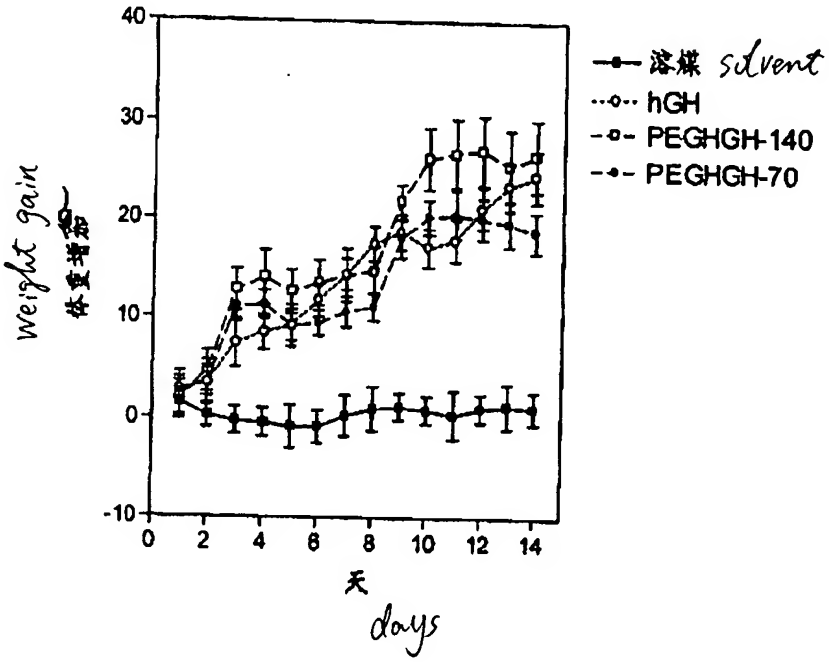
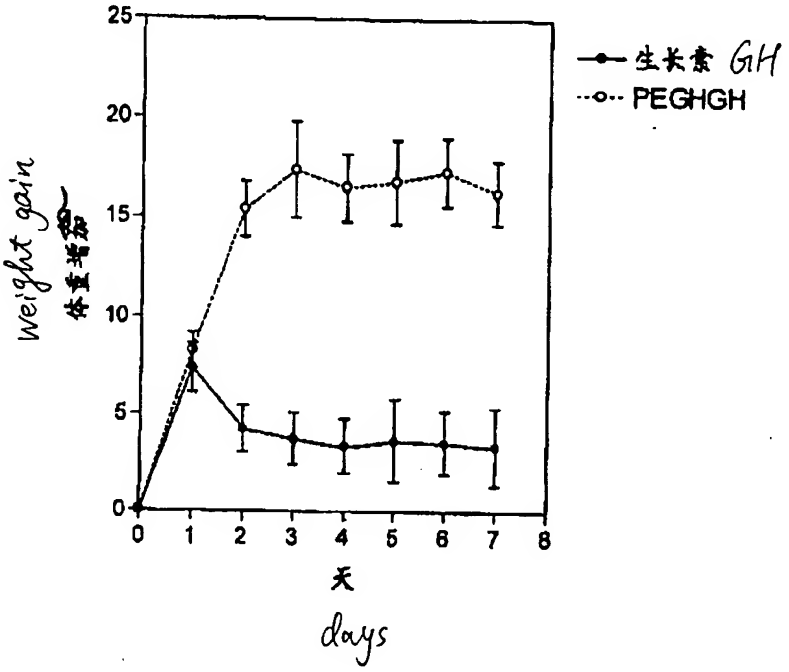

40
30
20
10
0
-10
0
2
4
6
8
10
12
14
Weight gain
体重增加
天
days
溶媒 solvent
hGH
PEGHGH-140
PEGHGH-70

图 6



Appl. No.03133278.1 Publ. No. CN 1477126A
Applicant: Jinsai Pharmaceuticals Ltd, Changchun, China
Co applicants: Li Weihua; Dong Jian; Wang Shaobai
Inventors: Li Weihua; Dong Jian; Wang Shaobai

Long-acting Growth Hormone and its Pharmaceutical Composition Field of the Invention

This invention belongs to the field of biological preparation, particularly relates to the long-acting growth hormone (GH), its preparation method, function and use.

Background of the Invention

Human growth hormone (h GH) is a protein hormone secreted by the human pituitary gland, and an indispensable regulating hormone for the human normal growth and development. The earliest source of GH is extracted from the human pituitary gland, and owing to the limited source and difficult to assure the quality, this product is hard to use extensively. The appearance of gene engineering makes the mass production of h GH possible, and the recombinant h GH has become the second product for the industry of bioengineering pharmacy. At present, It is mainly used in dwarfism, recovery of large-area wounds, adult growth hormonoprivia, recovery of AIDS emaciated etc.

The main route of h GH administration is limited to the frequent injection because of the short half-life (<2hr). The conventional method is daily injection, the period of admen is in six months to one year. The frequent injection makes serious inconvenient for the patient, and enhances the admn cost. Therefore, the important items to be settled urgently are: improving the route, reducing the frequency, & decreasing the cost of the GH admn, and in creasing the complying with the doctor, & the curative effect.

A slow releasing fine particle formed by embedding tech PLGA has been

jointly developed by Genetech Co and Alkermers Co, (US), approved by FDA, and appeared on the market. However, the production technology of this product is complicated and the curative effect is slightly inferior than the primary product.

The modification of protein by PEG has been found successful application for prolonging the half-life, reducing the immunogenicity and increasing the stability of the product. At present, the product using this tech and having been appeared on the market in clued: PEG-asparaginase, PEG-ADA, PEG-interferon's admn reduced from formerly injecting once per 2 or 1 days to injecting once per week, and the curative effect improved from injecting once daily to injecting once per chemotherapy course.

The modification of GH by PEG has been studied by Clark et al (J Bio Chem. 271:21969-21977,1996), and a product whose GH half-life is made to prolong to over 15hr has been obtained, the injecting frequency in a model of depituitary rats can be reduced to injecting once per 5 days. This study using PEG (5kDa), so the ideal effect of prolonging GH half-life can achieve only modification by multiple PEQ molecules, but an excessive modification would create relatively larger influence to the GH activity. This study indicates: the most ideal effect is the modification by 5 PEG molecules. though this method has proved the feasibility for modifying GH by PEG, the dose must be increased to compensate the decrease in activity, and the non-uniformity of the product makes the process and quality control difficult to assure. Therefore, the practicability of this study is weak.

Disclosure of the Invention

The object of this invention is to provide a method for modifying growth hormone with high-molecular-weight polyethylene glycol, there by to obtain a modified product capable of purloining the half-life of GH.

The technical solution if this invention is:

Polyethylene Glycol-Growth Hormone Conjugate & its Preparation

The in vivo half-life of GH depends on various aspects of factors, e.g. protein stability, conjugation & isolation of conjugated protein, non-specific adsorption & degradation, antibody formation, kidney elimination, conjugation & endolysis with acceptor etc. These factors determined the in vivo distillation, residence time & metabolism, while the pharmaco-kinetic characteristic of exogenous GH was determined. Conjugation of protein with a hydrophilic polymer e.g. PEG, increases the stability and reduces the non-specific adsorption & antigenicity of the protein, and considerably reduces kidney eliminating effect at the time of a fairly high-molecular-weight. So it is an effective method for prolonging the in vivo half-life of protein drug.

The MW of h GH is 20kDa, it has 10 primary aminos wherein one α-amino and 9 lysine-side-chain aminos are very active, and capable of reacting with activated carboxy to form peptide chain. Activating PEG molecule with N-hydroxysuccinamide (NHS) is a common method for conpling of macromolecule at present.

Clark et al (J. Biol. Chem., 271:21969-77, 1996)disclosed: more PEG (5 kDa) molecules are capable of coupling to h GH, and the MW increases, the half-life of conjugate prolongs along with the increase in the No. of PEG molecules coupled. However, a seriously negative effect on the bioactivity of GH was produced while more PEG molecules are coupled, the more the No. of PEG molecules coupled the more the loss of bioactivity. Clark's conclusion: the most preferred long-acting result being "each GH molecule coupling 5 PEG molecules".

The half-life of hGH depends on various aspects of factors, wherein the kidney elimination is a matter of importance. In order to reduce kidney elimination, the MW must be above a certain critical value (~70 kDa), hence the h GH must coupled with macromolecular PEG. And in order to reduce the loss of bioactivity, it would be best to couple only one PEG molecule. This

method has found a successful utilization in long-acting interferon (Bailon et al, Bio conjugate Chem., 12:195-202, 2001). For achieving "only one PEG molecule is coupled with one GH molecule", the fairly possible site is α-amino group, its pKa is 7.6~8.0 (lower than that of lysine-side-chain amino 10.0~10.2). The specific coupling of PEG to N-end of G-CSF protein can be achieved by coupling aldehyde group with α-amino group at a low pH (Kinstler at al., US 5,985,265, 1995). However, the coupling efficiency of GH is very low at said reaction conditions.

On the one hand, this invention provides a PEG-GH conjugate (mol ratio 1:1), on the other hand provides a suitable method for producing said conjugate by reacting of GH with the NHS activated PEG in a buffer solution.

The MW of NHS activated PEG can be 20 kDa~120kDa, more preferably 40 kDa~80 kDa. All the PEG can be linear, and branched as well. The activated branched PEG (40 kDa, mPEG 2-NHS) was bought from Shearwater Co, US, and the branched PEG (80kDa, mPEG4-NHS) & its activation are disclosed in this invention. Buffer system, pH, mole ratio, temperature & time of reaction are the important control conditions in the reaction process. Phosphate buffer is the first choice buffer system of this invention, the other buffer solutions can also be used provided they have sufficient buffer ability and not interfere with coupling. The concentration of the buffer solution is 50~200mM.

The pH of conventional NHS coupling buffer solution is controlled at 7.5~9.0 for increasing the coupling efficiency, the pH of our coupling buffer was reduced to 7.5~9.0, more preferably 6.5~7.0, for enhancing the selectivity. At such conditions, generally recognized as low efficiency. The mol ratio of PEG to GH can be 1:1~10:1, more preferably 3:1~7:1. The reaction pH produces a certain effect on the mole ratio, the higher the pH, the lower the mole ratio of PEG to GH. There exists a certain relationship between temperature & time of reaction, the reaction can be completed for 1 hr at

room temperature and for over night at 4℃. At our low pH condition, the aq. solution-stability of NHS active group increases greatly, so extending the time favored complete reaction. In addition, protein generally has a better stability at law temperature, hence the first choice condition is 2~8℃ overnight. After the above requirements being met, the main product is a conjugate wherein one PEG molecule is coupled with one GH molecule. Through the exact coupling site remains to be verified, the most possible site is the N-end of α-amino group.

Another aspect of this invention is to provide a method for purifying the PEG0Ghconjugate. The main component that must be isolated form the conjugate is the uncoupled GH, The difference between these two is one PEG molecule. Owing to PEG (20kDa), the conjugate is isolated from the uncoupled GH by Superdex 75(Pharmacia Biotech) MS column, the conjugate appears at evacuated volume, and the uncoupled GH has a longer retention time. There are no much limits to the buffer during chromatography, it can be adjusted according to the next process.

By this method, the conjugate can be effectively isolated from the uncoupled GH, but the free PEG can not be separated from the conjugate. This method can be used for other proteins similar in MW such as red cell growth factor, G-CSF, interferon, GM-CSF, interleukin etc. The requirements being met are: PEG's MW>20kDa, Protein's MW<40kDa.

The other aspect of this invention is to provide a method for isolating PEG-GH conjugate simultaneously from the uncoupled GH & PEG. This method is: first the coupled reaction product is displaced into a cationic buffer solution of lowest ionic strength e.g. Tris, then the reaction products are isolated in anion exchange column and eluted with the buffer solution of different ionic strength. In this condition, the free PEG is isolated first owing to its very low binding force, the PEG-GH conjugate is eluted at a lower ionic strength owing to its lower binding force compared with the free GH,and the

free GH is eluted at a higher ionic strength. More particularly, After the reaction has completed, firstly using desalting chromatography e.g. Sephadex G25, the buffer solution was displaced by a cationic buffer, e.g. Tris (pH>6.5), the concn generally being 20mM. Then the reaction mixture was purified using Q union exchange column (e.g. Mono-Q or Q-Sepharose, Pharmacia Biotech), the free PEG is isolated during applying and washing, and the PEG-GH conjugate and free GH are bound to the column. After applying, the reaction mixture was washed with the same balancing solution (20mM Tris, pH 7.4), and eluted with the same buffer solution while in creasing gradually ioniv strength. The binding force of PEG-GH conjugate with the cation exchange column is weakened, hence the PEG-GH conjugate is eluted in 50mM NaCl eluent (50mM NaCl, 20mM Tris, pH7.4), and the uncoupled GH is eluted in > 65 mM NaCl eluent (>65mM NaCl, 20mM Tris, pH7.4). The free PEG and GH can be effectively separated from the required coupling product, a high purity PEG-GH conjugate suitable for medicinal use is obtained.

1. Pharmaceutical composition

Removing rats pituitary results in artificial growth hormonoprivia. The bodyweight of depituitary rats will remain unchanged without the exogenous GH. This model is a standard method for detecting the bioactivity of GH. The in vivo half-life of hGH in rats is less than 30min (Jorgensen etal, Pharmacol Toxicol, 63:129-134, 1988). In order to observe the bioactivity of GH, hGH must be injected daily into the rats. By "long-acting effect", we mean "the bioactivity of GH has prolonged" which can not be obtained by merely dose-increase. For example, in the depituitary rats, the extent of weight gain can be correspondingly increased by increasing the GH dose, but regardless of the dose, its weight effect not exceed 24hr. The long-acting GH must achieve the same goal of weight gain by the same or less dose at reduced frequency of admn.

This invention has provided a method for curing different diseases of mammal. The method includes, an effective dose of PEG-GH conjugate is provided for the mammal to be cured. This conjugate can be used to cure the diseases of growth hormonoprivia or the beneficial-effect-producing while GH used. The does of PEG-GH conjugate generally depends on the GH activity contained in the conjugate. The maximum does depends on that no main lineal side effects results. Generally, the does of conjugate used can refer to the accumulated routine GH dose per unit time. This reference does is only used for illustration, the selection of the optimal dose must be determined by clinical symptoms.

The high purity PEG-GH conjugate of this invention can be used in producing the pharmacautical composition for curing mammal, these pharmaceutical composition can be solution, suspension, lyophilized powder, tablet, capsule etc. The route of administration is mainly parenteral, but oral or inhalational can also be used.

2. Synthesis of HMW Branched PEG

The other aspect of this invention is to provide a method for synthesis of high-molecular-weight branched PEG. The molecular structure of this PEG is defined as Rx-B-R1. Wherein R is a PEG branched chain linked to the branched linker B, which can be a linear molecule or branched molecule. x is the number of PEG branched chin linked to branched linker, which can be 2~4. The size & shape of PEG branched chain can be the same or different and of 2kDa~20 kDa MW. B is a branched linker having at least two nucleophilic groups and adjacent to PEG branched chain through the reaction of muclcophilic group, and linked to another PEG branched chain R1. The nuileophilic group can be an amino group, and the other can be carboxy or hydroxy. Lysine is a typical sample of linker B.

R1 is a linear PEG molecule having different functional groups, wherein one end linking to B, another is an afteractivable group, e.g. carboxy or

hydroxy, and of 2kDa~20kDa MW.

During synthesis, firstly the branched chain R1 is reacted with activated B to form branch, the nucleophilic groups of reactant B are protected by the same or different protecting groups. As for lysine, the PEG molecule of the same size or shape can be linked while using the same protecting groups [e.g. F_{moc}-Lys(F_{moc}) or B_{oc}-Lys(B_{oc})] after deportation; the PEG molecules of different size or shape can be linked while using different protecting groups [e.g. F_{moc}-Lys(B_{oc})] or opposite protection. To form a p-nitrophenyl or succinimidyl group is adopted for the activation of B, and reacted with the nucleophilic group R1.

After the reaction has completed and deprotected, the structure of the product B-R1 has two nucleophilic groups (amino) at one end, and carboxy or hydroxy group at another for the final activation.

The PEG branched chain R generally is a methoxy PEG, and can be activated with conventional method. The activated PEG can also be bought from shearwater Co. The PEG activated by p-nitrophenyl or NHS can be reacted with the nuileophilic group (amino) to form a stable final product covalently bonded to the nucleophilic groups (amino) on B-R1 $-R_x$-B-R1.

Compared with the other method for preparing branched PEG (Monfardini et al, Bioconjugate chem, 6:62-69, 1995; Nathan et al, Macromolecules, 25:4476-84, 1992), the advantage of this invention is, all the reaction being carried out in organic solvent, and good for the increase in reacting efficiency, having space between reactive site and macromdecule, good for completing reaction, while the number, size and shape of side chain can be free to choose.

This invention discloses a method for modifying GH by macromolecular weight PEG, and a modified product of prolonging markedly the half-life of GH. Said modified product has simple structure, uncomplicated production technique, strict quality control and strong practicability.

Brief Description of the Drawing

Fig.1 Weight gain effect of PEG-hGH conjugate in depituitary rats

■Gp solvent (AQ buffer) ●Gp 40N-70 [70μg PEG (40kD)-GH]

□Gp GH (10μghGH) ○Gp 80N-70 [70μg PEG (80kD)-GH]

Daily mean weight gain after cure (mean value ± std variance)

Fig 2. Volume-effect relationship of PEG-hGH conjugate in depituitary rats

■Gp solvent (AQ buffer) ●Gp 80N-30[30μg PEG (80kD)-GH]

□Gp GH (10μg hGH) ○Gp 80N -70[70μg PEG (80kD)-GH]

Daily mean weight gain after cure (mean value ± std variance)

Fig.3 SDS-PPA gel electrophoresis of PEG-hGH conjugate

Streak 1 hGH

Streak 2 hGH:PEG (1:3) react prod

Streak 3 hGH:PEG(1:5) react prod

Streak 4 hGH:PEG(1:7.5) react prod

Streak 5 hGH:PEG(1:10) react prod

Streak 6 LMW standard of protein

streak 7 {Mono – Qcol, 50mMNaCl} peak (hGH:PEG 1:3)

streak 8 {Mono – Qcol, 65mMNaCl} peak (hGH:PEG 1:3)

streak 9 {Mono – Qcol, 500mMNaCl} peak (hGH:PEG 1:3)

Fig.4 SDS-PPA gel electrophoresis of PEG-hGH conjugate

streak 1 {Mono – Qcol, 50mMNaCl} peak (hGH:PEG 1:5)

streak 2 {Mono – Qcol, 65mMNaCl} peak (hGH:PEG 1:5)

streak 3 {Mono – Qcol, 500mMNaCl} peak (hGH:PEG 1:5)

streak 4 {Mono – Qcol, 50mMNaCl} peak (hGH:PEG 1:7.5)

streak 6 {Mono – Qcol, 500mMNaCl} peak (hGH:PEG 1:7.5)

streak 7 {Mono – Qcol, 50mMNaCl} peak (hGH:PEG 1:10)

streak 8 {Mono – Qcol, 65mMNaCl} peak (hGH:PEG 1:10)

streak 9 {Mono – Qcol, 500mMNaCl} peak (hGH:PEG 1:10)

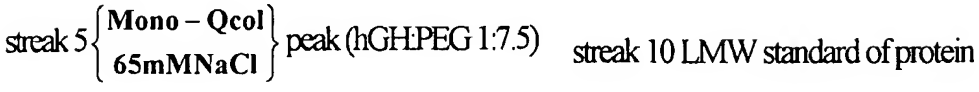


Fig.5 Weight gain effect of hGH and PEG(40kDa)-hGH conjugate in depituitary rats

■Gp solvent (AQ buffer) ●Gp 70μg PEG (40kDa)-hGH

○Gp GH (20μg) □Gp 140μg PEG (80kDa)-hGH

Daily mean weight gain after cure (mean value ± std variance)

Fig.6 Weight gain effect of hGH and PEG-hGH conjugate in depituitary rats (same dose once injected)

●Gp hGH (140μg) ○Gp 140μg PEG (40kDa)-hGH

Daily mean weight gain after cure (mean value ± std variance)

Preferred Embodiments of the Invention

Example 1 Preparation of HMW Branched PEG

For prolonging the half-life of the growth hormone, the high-molecular-weight (HMW) branched polyethylene glycol (PEG) is relatively suitable for use. Described below is a method for synthesizing 80kDa multibranched PEG, which can be used for synthesizing other PEG of different MW and shape as well.

11.8mg of Fmoc-Lys (Fmoc)-OH (MW 590, 20μmol, Advanced ChemTech) was dissolved in 120μl of dimethylformamide (DMF) containing 24μmol of N-hydroxysuccinimide (NHS, Sigma), 3.8 μl of diisopropyl carbodiimide (DIC, MW 126, d=0.8, 24μmol, Advanced Chem Tech) was added, the reaction mixture was shook at room temperature for 2hr. 68mg of NHz-PEG-COOH (MW 3400, 20μMOL, Shearwater) was dissolved in 100μl of dichloromethane (DCM), 6.9μl of diisopropyl ethylamine (DIPEA, MW

129, d=0.74, 40μmol, Sigma) was added, and mixed with the forepart reaction. After shaking at room temperature for 1hr, 10% hexahydropyridine was added, shook at room temperature for 5min, the reaction product, at the volume ratio of mixture to ether >1:10, was precipitated from ethyl ether and vacuum dried to obtain the final product $(NH_2)_2$-PEG-COOH (78mg).

8.8mg of $(NH_2)_2$-PEG-COOH (5μmol, amino-group) was dissolved in 100μl of DCM, 200mg of m PEG_2 40K NHS (MW 42500, 5μmol, Shearwater) and 1.7μl of DIPEA (10μmol) were added, the reaction mixture was shook at room temperature overnight. The product was precipitated from ethyl ether and vacuum dried.

The synthesized PEG (80kDa) was applied to Superdex 200 chromatographic column (2.6×40cm), and purified. Balancing the column with water, and the product to be purified was dissolved in 5ml of water. After applying, the product was eluted with water and observing at 214nm, the first peak was mixed and vacuum-dried.

The purified 80kDa PEG was activated with DIC. 100mg of 80kDa m PEG_4-COOH (1.25μmol) was dissolved in 0.5ml of dry DCM, 1.3μmol of NHS and 0.25μl of DIC (1.5μmol) were added. The reaction mixture was shook at room temperature overnight. The product was precipitated from ethyl ether, vacuum-dried, and dry preserved at -20°c.

Example 2 Conjugating of PEG (80Da & 40kDa) to GH

234mg of GH (lyophilized powder) was dissolved in 4ml of 0.1M Na phosphate solution (pH 7.0). Hi Trap Sephadex G25 desalting column (2.5×15cm, Pharmacia Biotech) was balanced with 0.1M Na phosphate solution (pH 7.0), and the GH sample was applied, observed at 280nm, and the first peak collected (18ml). The light density at 280nm was determined; the GH concentration was calculated with the extinction coefficient. The result: light density 1.2, protein concn 1.76mg/ml, total protein 32mg.

During conjugating of PEG (80kDa), 60mg of activated m PEG_4

(80kDa)-NHS (0.7μmol) was added into 1.1ml of Na phosphate solution [pH 7.0, containing 6mg (0.3μmol) of GH], the reaction mixture was shook at 4°c overnight.

During conjugating of PEG (40kDa), 60mg of activated m PEG_2 (40kDa)-NHS (1.5μmol) was added into 2ml of Na phosphate solution [pH 7.0, containing 10mg (0.3μmol) of GH], the reaction mixture was shook at 4°c overnight.

Example 3 Purification of PEG (80kDa & 40kDa)-GH Conjugate

Using Superdex 75 Chromatographic column (1.5×40cm). (Pharmacia Biotech), purified PEG (80kDa & 40kDa)-GH conjugate was obtained. The chromatographic column was balanced with a solution (pH 6.0, containing 150mM Nacl, 10mM of Na citrate, 0.2% Tween 20), the volume of applying was lower than 5% that of the column. The protein peak was observed at 280um, and the first elution peak was collected:

PEG (40kDa)-GH conjugate: 35ml, 0.16mg/ml, tot protein 5.6mg

PEG (80kDa)-GH conjugate: 28ml, 0.08mg/ml, tot protein 2.6mg

Example 4 Long-acting Effect of the Conjugates in Depituitary Rats

60 depituitary femal rats (8wk-age, Sprague Dawley) were bought from Taconic Farms Co [wherein 4 rats being rejected for poor health and 3 rats for the excess wt gain (> one std variance)], the rest 53 rats were randomly classified as:

Gp solvent: 10 rats were subcutaneously injected daily for 14 days with 1ml of AQ buffer solution (0.2% Tween-20, 150mM NaCl, 10mM Na citrate, pH 6.0);

Gp GH: 10 rats were subcutaneously injected daily for 14 days with 1ml of AQ buffer solution (coutaing 10μg h GH);

Gp 80N-70: 11 rats were subcutaneously injected at first and eighth days with 1ml of AQ buffer solution [containing 70μg PEG (80kDa)-GH

conjugate];

Gp 80N-30: 11 rats were subcutaneously injected at first and eighth days with 1ml of AQ buffer solution [containing 30μg PEG (80kDa)-GH conjugate];

Gp 40N-70: 11 rats were subcutaneously injected at first and eighth days with 1ml of AQ buffer solution [containing 70μg PEG (40kDa)-GH conjugate];

Assaying daily the body weight of the rats, a plot of weight change in cure course was shown in Fig 1 and Fie 2. the mean values of weight change for each group of rats at 7 and 14 days, and the statistic analysis were listed at Tab 1 and Tab 2. In statistic analysis, the t test function of EXCEL software was used, the scale is two-tailed and in equal variance.

Tab1. Mean weight gain of the rats at 7 days after cure

Group	n	Wt gain (g) (mean±sd)	P(solvent)	P(GH)
Solvent	10	0.6±1.8		
GH	10	14.2±2.1	<0.0001	
40N-70	11	13.0±2.2	<0.0001	>0.05
80N-30	11	9.9±2.2	<0.001	<0.01
80N-70	11	13.2±2.0	<0.0001	>0.05

Tab2. Mean weight gain of the rats at 14 days after cure

Group	n	Wt gain (g) (mean±sd)	P(solvent)	P(GH)
Solvent	10	1.0±2.7		
GH	10	19.8±2.8	<0.0001	

40N-70	11	20.0±3.2	<0.0001	>0.05
80N-30	11	14.5±4.1	<0.0001	<0.01
80N-70	11	19.5±4.4	<0.0001	0.05

The result indicates: the mean weight gains of Gp 40N-70 & 80N-70 were not different from that of Gp GH (positive control), i.e. the high-molecular-weight branched PEG-GH conjugate (once per 7 days) has produced the same weight gain effect as the conventional GH (once daily). There exists a volume-effect relationship between the weight gain effect and the dose of conjugate.

Example 5 Conjugating of PEG to GH & Purification

43.5mg of hGH (lyophilized powder) was dissolved in 1.0ml of water, and applied to Hi Trap G25 desalting column, the buffer solution was displaced by 50mM Na phosphate (pH 6.5). Light density was determined at 280nm, and protein concn was calculated with extinction coefficient (0.68). the total amount of recovery GH (5.7mg) was divided into four portions (each containing protein 1.4mg volume 0.75ml).

Four portions of appropriate amount of m PEG2-NHS (MW 40kDa, shearwater) were added separately, according to the following table, into the GH samples, the reaction was in progress at 4°c overnight.

PEG:Hgh(molratio)	3:1	5:1	7.5:1	10:1
hGH (nmol)	71.62	71.62	71.62	71.62
PEG (mg/nmol)	8.6/214.9	14.3/358.1	21.4/537.2	28.6/716.2

After the above reaction has compelled, using Hi Trap G 25 desalting column, the buffer solution was displaced by 20mM Tris (pH 7.4). Each sample was applied to the balanced Mono-Q HR 5/5 chromatographic column

(Pharmacia Biotech), and eluted by gradient gain in ion strength, the elution peak was observed at 280nm.

Eluting solution 1: 50mM NaCl, 20mM Tris, pH 7.4;

Eluting solution 2: 65mM NaCl, 20mM Tris, pH 7.4;

Eluting solution 3: 500mM NaCl, 20mM Tris, pH 7.4;

To 80μl of each elution peak's sample, 5μl×5 of SDS-poly propenamide gel sample treated buffer solution was added, and heated at 100°c until the total volume was less than 30μl. All the samples were applied to 4-20% prepared SDS-polypropenamide gel electrophoresis. Fig 3 and Fig 4 are the photographs after electrophoresis.

The result indicates, a conjugate wherein one PEG molecule is coupled with one GH molecule, can be made effectively from GH and m PEG2-NHS. The efficiency of conjugation is higher when the mol ratio of GH to PEG is 1:5~1:7.5, and fewer phenomenons of more PEG molecules coupled with one GH molecule exist, this result is the best.

Using anion-exchange column Mono-Q, the PEG-GH conjugate can be isolated effectively from the free GH and free PEG. The free PEG got isolated as soon as applied and eluted with balance solution, the PEG-GH conjugate was eluted in 50mM NaCl eluent, and the free GH was eluted in >65mM NaCl eluent. All of 50mM NaCl elution peaks eluted from Mono-Q column were mixed, and said buffer was exchanged, with Hi Trap G 25 desalting solumn, into AQ buffer (150mM NaCl, 0.2% Tween-20, 10mM Na citrate, pH 6.0). The buffer exchanged sample was aseptic filtered (CAMEO 25AS) by 0.22μm filter, and preserved at 2~8°c for bioactivity measurement.

Example 6 Preparation of PEG-GH Conjugate Using Anion-exchange Column

88.4mg of h GH (lyophilized powder) was dissolved in 10ml of water,

and applied to two connected Hi Trap G25 desalting column, the buffer was displaced by 50mM Na phosphate (pH 6.5). Light density was determined at 280nm, and protein concn calculated with extinction coefficient (0.68). the total amount of recovery GH (14mg, 5.5ml) diluted with 50mM Na phosphate solution (pH 6.5) to 8ml, and was divided into eight portions (each containing protein 1.75mg, volume 1 ml).

Eight portions of m PEG2-NHS (MW 40kDa, shearwater) were added separately, according to the following table, into the GH samples the reaction was in progress at 4°c overnight.

PEG:hGH (mol ratio)	7.5:1
HGH (nmol)	87.5/set
PEG (mg/nmol)	26.25/656.25/set

After the above reaction has completed, using Hi Trap G25 desalting column, the buffer solution was displaced by 20mM Tris (pH 7.4). Each sample was applied to the balanced Mono-Q HR 5/5 chromatographic column (Pharmacia Biotech), and eluted by gradient gain in ion strength, the elution peak was observed at 280nm.

Eluting solution 1: 50mM NaCl, 20mM Tris, pH 7.4;

Eluting solution 2: 65mM NaCl, 20mM Tris, pH 7.4;

Eluting solution 3: 500mM NaCl, 20mM Tris, pH 7.4;

To 80μl of each elution peak's sample, 5μl×5 of SDS-polypropenamide gel sample treated buffer solution was added, and heated at 100°c until the total volume was less than 30μl. All the samples were applied to 4~20% prepared SDS-poly propenamide gel electrophoresis, and photographed after electrophoresis.

The result indicates, the isolating effects of anion-exchange column Q-Sepharose Fast Flow and Mono-Q column are the same, PEG-GH conjugate was eluted in 50mM NaCl eluent.

All of 50mM NaCl elution peaks eluted from Q-Sepharose column were mixed, and said buffer was exchanged, with Hi Trap G25 desalting column, into AQ buffer (150mM NaCl, 0.2% Tween-20, 10mM Na citrate, pH 6.0). the buffer exchanged sample was aseptic filtered (CAMEO 25AS) by 0.22μm filter, and preserved at 2~8°c for bioactivity measurement.

Example 7 Bioactivity of PEG-GH Conjugate in Depituitary Rats

40 depituitary female rats (8 wk-age, Sprague Dawley) were bought from Taconic Farms Co [wherein 2 rats being rejected for poor health and 3 rats for the excess wt gain (> one std variance)], the rest 35 rats were randomly classified as:

Gp solvent: 9 rats were subcutaneously injected daily for 14 days with 1ml of AQ buffer solution (0.2% Tween-20, 150mM NaCl, 10mM Na citrate, pH 6.0);

Gp GH: 6 rats were subcutaneously injected daily for 14 days with 1ml of AQ buffer solution (containing 20μg h GH);

Gp PEG-h GH-70: 10 rats were subcutaneously injected of first and seventh days with 1ml of AQ buffer solution [containing 70μg PEG (40kDa)-GH conjugate];

Gp PEG-h GH-140: 10 rats were subcutaneously injected of first and seventh days with 1ml of AQ buffer solution [containing 30μg PEG (80kDa)-GH conjugate];

Assaying daily the body weight of the rats, a plot of weight change in cure course was shown in Fig 5. The mean values of weight change for each group of rats at 7 and 14 days, and the statistic analysis were listed at Tab 3 and Tab 4. In statistic analysis, the t test function of EXCEL solfware was used, the scale is two-tailed and inequal variance.

Tab 3 Mean weight gain of the rats at 7days after cure

Group	n	Wt gain (g) (mean±sd)	P(solvent)	P(GH)
Solvent	9	1.93±2.10		
GH	6	17.07±1.94	<0.0001	
PEGhGH-70	10	12.99±2.66	<0.001	<0.05
PEGhGH-140	10	16.21±1.56	<0.001	>0.05

Tab 4 Mean weight gain of the rats at 14days after cure

Group	n	Wt gain (g) (mean±sd)	P(solvent)	P(GH)
Solvent	9	2.77±1.66		
GH	6	27.12±2.95	<0.0001	
PEGhGH-70	10	20.79±3.00	<0.0001	<0.001
PEGhGH-140	10	28.59±2.81	<0.001	>0.05

The result indicates: 140μg of PEG-GH conjugate (Gp PEG-hGH-140, once per 7 days) has produced the same weight gain effect as 20μg of the conventional GH (once daily). There exist a volume-effect relationship between the weight gain effect and dose of conjugate.

Example 8 Pharmacodynamics of PEG-GH compared to GH In Once Administered Depituitary Rats

9 depituitary female rats (11wk-age, Sprague Dawley) were bought from Taconic Farms Co, and randomly classified as:

Gp GH: 4 rats were once subcutaneously injected with 1ml of AQ buffer

solution (containing 140μg/ml h GH);

Gp PEG-hGH: 5 rats were once subcutaneously injected with 1ml of AQ buffer solution (containing 140μg PEG-hGH conjugate). A plot of daily weight change was shown in Fig 6, and the mean weight gains of the rats at 7 days were listed at Tab 5.

Tab 5 Mean weight gain of the rats at 7 days after cure

Group	n	We gain (g) mean±sd	P
GH	4	3.43±1.79	
PEG-Hgh	5	16.28±1.22	<0.0001

The result indicates: the weight gain of Gp PEG-hGH (140μg) was markedly above that of Gp GH (140μg), i.e. PEG-hGH has attained the long-acting object, the same result is unable to achieve by merely increasing the GH dose.

Claims

1. A high purity polyethylene glycolylated human growth hormone (PEG-GH) conjugate, wherein the mol ratio of PEG to GH is 1:1.

2. The conjugate of Claim 1, wherein the molecular weight (MW) of PEG is 20kDa~120kDa.

3. The conjugate of claim 2, wherein the MW of PEG is 40kDa~80kDa.

4. The conjugate of claim 1, wherein the PEG molecule contains 2~4 branched side chain.

5. The conjugate of claim 4, wherein the MW of each PEG side chain is 5kDa~20kDa.

6. The conjugate of claim 1, wherein the purity of conjugate is greater than 95%.

7. The conjugate of claim 1, wherein the conjugate can take a long-acting effect.

8. A method for producing the conjugate of claim 1~7, characterized by couplings of monofunctionally activated PEG to GH at pH 5.5~7.5 of aqueous solution.

9. The method for producing the conjugate of claim 8, characterized by coupling of monofunctionally activated PEG to GH at pH 6.5~7.0 of aqueous solution.

10. The method for producing the conjugate of claim 1~7, characterized by isolating and purifying the coupled mixture using MS chromatography (Superdex 75).

11. The method for producing the conjugate of claim 1~7, characterized by isolating and purifying the coupled mixture using an anion-exchange chromatographic column.

12. The method of claim 11, wherein the anion-exchange column is Q.

13. The method of claim 12, wherein the conjugate of claim 1~6 is eluted in 50mM NaCl buffer solution.

14. A pharmaceutical composition containing the conjugate of claim 1~7, and pharmacentically acceptable dilutent, stabilizer, and adjutant.

15. The parmacentical composition of claim 14, characterized by said composition having a long-acting effect.

16. A high molecular weight branched PEG molecule, said molecular structure is defined as: Rx-B-R1 wherein R being a PEG branched chain linked to linker B, x being the No. of branched chains (2~4), R1 being a difunctional PEG molecule wherein one end linking to B, another having free carboxy or hydroxyl group.

17. A high molecular weight branched PEG molecule, its molecular weight is 20kDa~120kDa.

18. The high molecular weight branched PEG molecule, its molecular weight is 40kDa~80kDa.

19. A PEG conjugate, characterized by said conjugate is produced from the PEG molecule of claim 16~18 with the molecule of protein and other.

Abstract

This invention discloses a method for modifying growth hormone with macromolecular weight polyethylene glycol, thereby to obtain a modified product capable of prolonging markedly the half-life of the hormone. Said product has simple structure, uncomplicated production technology, strict quality control, and strong practicability.